Study on the Interaction Mechanism between Phospholipase A1 from Serratia marcescens and Its Auxiliary Proteins Based on Molecular Docking

Zhou Jie[1], Yang Meng[1], Liu Yan[1], Zhang Huimin[1], Li Chuang[1], Liu Qingtao[1], Wang Zhou[1], and Xue Zhenglian[1]

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Abstract

Previous studies showed a sequence encoding an auxiliary protein (PlaS) downstream of the phospholipase A1 (PlaA1) gene of *Serratia marcescens*. There is an interaction between PlaA1 and PlaS, which may be closely related to the high enzymatic activity property of phospholipase A1. In order to further investigate the interaction mechanism, it is necessary to explore binding sites of the interaction between PlaA1 and PlaS and the regulatory mechanism for enzymatic properties by molecular docking and site-directed mutagenesis. The results showed that the active center site of PlaA1 was encapsulated internally, and a "catalytic pocket" was formed externally by Leu197-Ser249. The docking process of PlaA1 and PlaS involved 29 and 30 amino acids, respectively, of which Phe186 and Lys238 of PlaA1 are involved in forming π-bonds and multiple hydrogen bonds. Therefore, Phe186 and Lys238 were site-directed mutated to Ala to obtain the mutant enzymes PlaA1 ^{F186A} and PlaA1 ^{K238A}, respectively. The results showed that the mutant enzymes showed no significant changes in optimum temperature and pH but poor stability. The kinetic parameters indicated that the affinity between PlaA1 and substrates was weakened, and the catalytic efficiency was reduced after mutation. Therefore, it demonstrated that Phe186 and Lys238 of PlaA1 provided non-covalent bonds conducive to the enzymatic activity and stability in the interaction between PlaA1 and PlaS, which would provide some theoretical basis for further rational design and modification of phospholipase A1 subsequently.

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Abstract

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Keywords: Phospholipase A1; Auxiliary protein; Molecular docking; Site-directed mutagenesis; Enzymatic properties

Introduction

Phospholipase A1 (EC3.1.1.3) is a class of carboxylate hydrolases with lipase properties that exclusively hydrolyze phospholipid sn-1 position acyl groups to produce free fatty acids and lysophosphorus [1]. Hemolytic phospholipids are excellent emulsifiers for industrial applications such as food technology, cosmetics, and pharmaceutical industries [2]. Phospholipase A1 is present in cells and tissues of many organisms. For example, it has been detected in bovine testis, human brain, rat liver, *Serratia marcescens* , wasp venom, and arabidopsis thaliana [3-9]. Phospholipase A1 can combine with the cell membrane to form membrane protein and be secreted out of the cell in vivo, playing an essential physiological function for organisms [10]. Bacterial phospholipase A1 is mainly found in the outer membrane and cytoplasm and is involved in protein transport, while in plants and animals, it is mainly found in the cytosol [11-14]. Phospholipase A1 is also a member of the triacylglycerol lipase family and has similar conserved sequences to human liver and pancreatic and guinea pig pancreatic lipase-related proteins, both with the typical "Ser-His-Asp" catalytic triad [14-16].

Currently, research on phospholipase A1 has focused on molecular modification and optimization of fermentation process conditions to improve the catalytic activity of phospholipase A1. Since the discovery in 1999 of *plaS* , a gene that overlaps 5bp downstream of the gene encoding phospholipase A1 (*plaA1*), researchers have found that*plaS* is not enzymatically active and that expression of*plaA1* alone produces a large number of inclusions without phospholipase activity, while co-expression of *plaA1* with*plaS* in *E. coli* produces active phospholipase A1 extracellularly. It indicates that PlaS has a regulatory effect on the extracellular expression of phospholipase A1.

Only our laboratory has researched the interaction between PlaA1 and PlaS worldwide so far. It has been shown that PlaS is an anchorin protein and belongs to the ANK2 family. According to the analysis of GenBank data (Protein-ID: AFN44705.1), its protein structure is composed of three regions, N-terminal, anchorin region, and C-terminal, where the anchorin region contains four typical ankyrin repeats (ANK repeat). Ankyrin is an intracellular linker protein, a single-stranded polypeptide that mediates protein-protein interactions. ANK repeats are found in proteins in many organisms, and their functions include cell signaling, maintenance of cytoskeletal integrity, regulation of the cell cycle, inflammatory response, development, and various transporter roles [17]. An isolated ANK repeat cannot perform any function, it must be linked by at least two consecutive repeats for the peptide chain it encodes to produce a standard fold [18-19]. It means that the number of ANK repeats in tandem has an enormous impact on mediating protein-protein interactions. It has not been reported whether anchorin function with enzymatic properties, which is also consistent with the lack of enzymatic activity of phospholipase A1 auxiliary protein PlaS. However, studies on whether the auxiliary or anchorin affects phospholipase A1 enzymatic activity are still unclear. It is still necessary to obtain more details on the mechanism of interaction between PlaA1 and PlaS.

Therefore, it is essential to explore the interaction mechanism of phospholipase A1 and its auxiliary protein by three-dimensional structural simulation and molecular docking, including prediction of key amino acids

and site-directed mutagenesis. Consequently, we aimed to find the confirmation of the key amino acids in the interaction between PlaA1 and PlaS and their effect on the enzymatic properties of phospholipase A1. All this research contributes to the further understanding of the molecular mechanism of PlaS in the regulation of phospholipase A1.

Methods

Strains

BL21(DE3), plasmid vector pET-28a(+), BP_{28} is the full-length gene of phospholipase A1 expressed in *E. coli*BL21(DE3) after mounting on pET-28a(+), which is an engineered strain constructed and preserved in our laboratory.

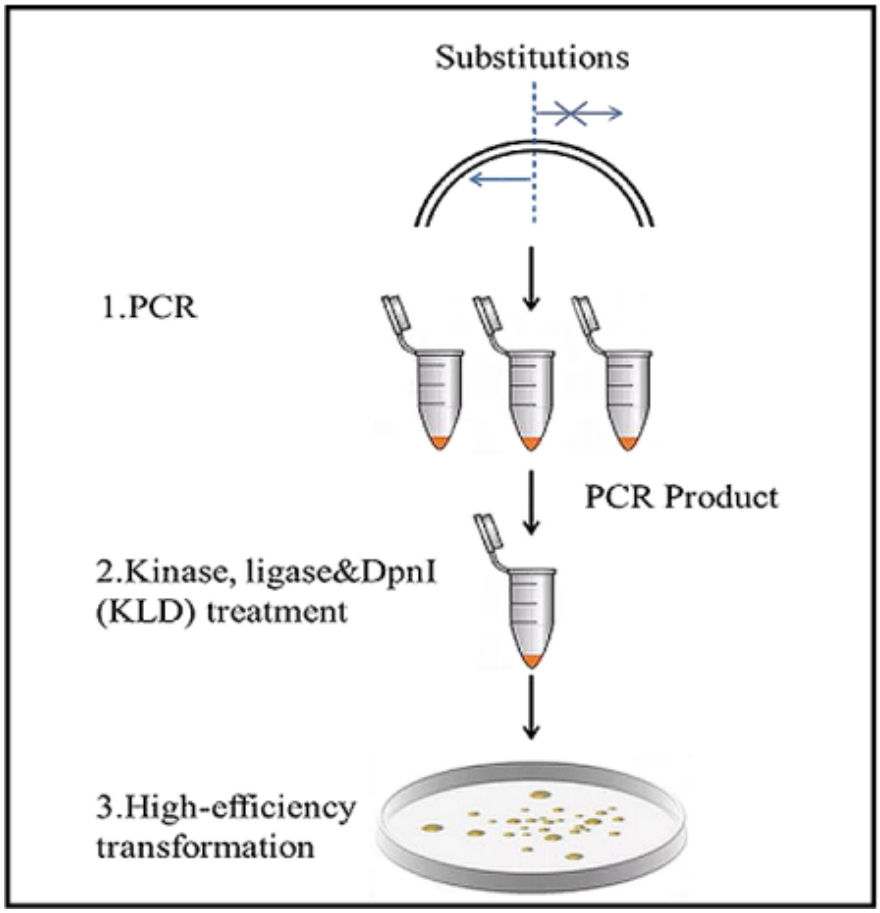
Amino acids sequences analysis and Homologous modeling of PlaA1 and PlaS

The amino acid sequence of PlaS published in GenBank (No: AFN44705.1) was analyzed online at the NCBI website (https://www.ncbi.nlm.nih.gov/) using the online software NPS@SOMPA (https://npsa-prabi.ibcp.fr) to analyze the secondary structure; DoGSiteScorer (https://proteins.plus/#dogsite) to calculate and analyze the protein catalytic pocket; ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) was used for online amino acid sequence comparison. The fold recognition method was used to predict the tertiary structure of PlaA1 and PlaS, and homologous modeling was carried out.

Molecular docking

The docking calculation of protein is realized by using ZDOCK in Discovery Studio 3.5.

Site-directed Mutagenesis



According to the analysis results of bioinformatics software, the binding site of PlaA1 and PlaS was selected for site-directed mutagenesis. The principle of site-directed mutagenesis is shown in Figure 1. Primers were designed to perform mutation on Phe186 and Lys238 in PlaA1. The PCR products were subjected to KLD reaction according to the following reaction system. After mixing, stand at room temperature for 5min. The target gene was introduced into competent cell BL21(DE3), and the mutant strain was obtained after screening and verification. The strain was named PlaA1^{F186} before the mutation and PlaA1^{F186A} after mutating PlaA1^{Phe186} to Ala. The strain was named PlaA1^{K238} before the mutation and PlaA1^{K238A} after mutating PlaA1^{Lys238} to Ala.

Fig. 1: Schematic diagram of site-directed mutagenesis principle.

Preparation of extracellular enzyme solution

The positive transformant was inoculated into LB broth containing 50ppm kanamycin, cultured overnight in a shaker at 37 and 200 r/min, and then inoculated into 100mL lactose self-induction fermentation medium containing 50ppm kanamycin (yeast powder 5g, tryptone 10g, lactose 5g, disodium hydrogen phosphate 0.025M) according to 1% of the inoculated amount after activation. 0.025M sodium phosphate, 1mmol magnesium sulfate, 7.5g glycine, 0.8g glucose, distilled water volume to 1000 mL, 115 sterilization 15min), under the condition of 37, 200 r/min oscillation culture for 8 h. The fermentation liquid after the fermentation endpoint was centrifuged at 12000rpm for 2min at 4, and the supernatant was extracellular enzyme liquid.

Enzyme activity determination

The activity of phospholipase A1 is defined as the amount of enzyme required to hydrolyze lecithin to produce 1μmol of free fatty acid per minute at 45 as one enzyme activity unit (U). Enzymatic specific activity was defined as the content of phospholipase A1 per milligram of protein. The protein content was determined using Bradford quantitative method, and the protein standard curve was drawn. After reacting the extracellular enzyme solution with Coomassie brilliant blue solution, the absorbance value was measured at 595nm, and the available protein content was calculated according to the protein standard curve. According to the enzyme activity, the specific enzyme activity was calculated.

Studies on enzymatic properties

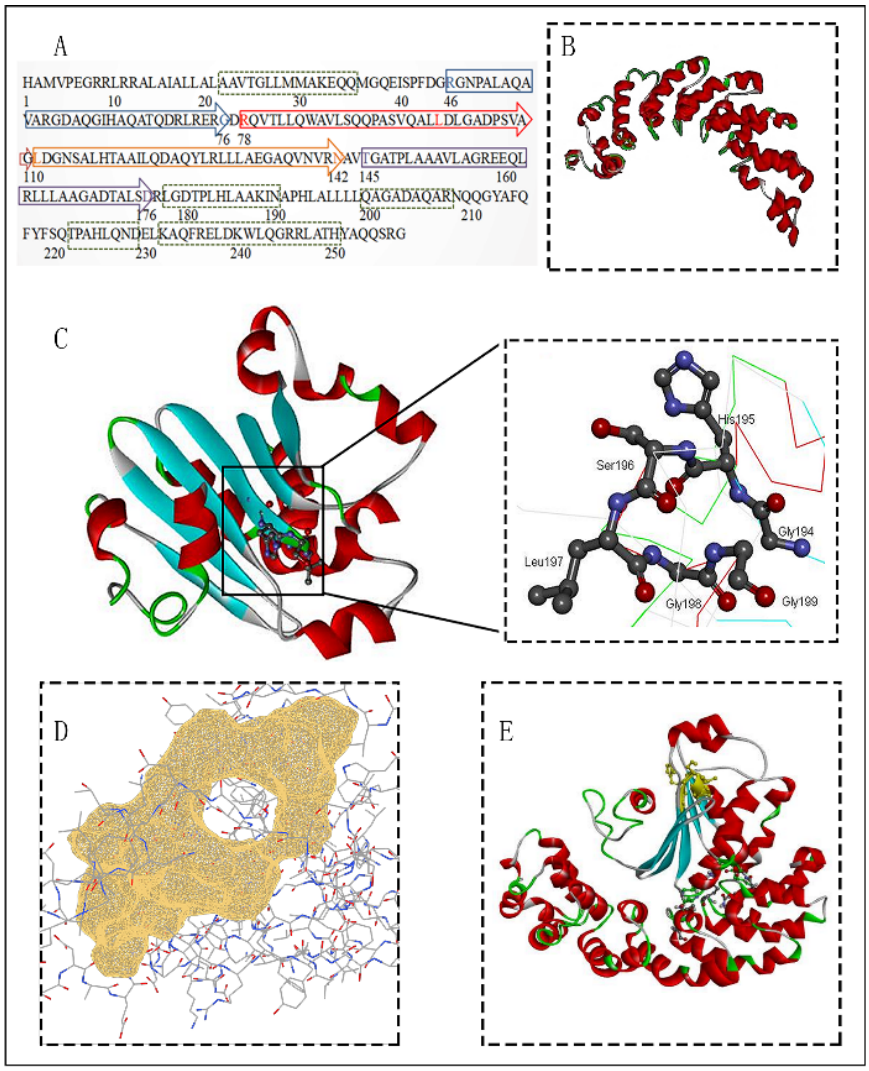
According to the method for determining the enzyme activity of phospholipase A1, other enzymatic reaction conditions were kept unchanged to determine the optimal temperature and optimal pH of phospholipase A1 from each mutant strain. The activity of phospholipase A1 produced by the starting strain BP_{28} was defined as 100% under the optimal conditions, and the relative enzyme activities of other samples were calculated.

Kinetic parameter

Lecithin was used as the substrate to react with 4%, 6%, 8%, and 10% substrate concentration at pH=6 and 45 for 15min, and the specific enzyme activity was measured. Reaction rate [V] was the ratio of specific enzyme activity to reaction time, with the reciprocal of reaction rate and substrate concentration [S] as the coordinate, K_m and V_{max} were calculated according to the Lineweaver-Burk mapping method.

Results

Bioinformatics analysis result of PlaA1 and PlaS



The secondary structures of PlaA1 and PlaS mainly consist of α-helix and random coil. The proportions of α-helix and the random coil of PlaA1 were 38.75% and 35.94%. The proportion of α-helix and the random coil of PlaS were 58.57% and 35.94%. The analysis showed that phospholipase A1 had no transmembrane helix structure, while PlaS 7-27AA had a transmembrane structure. Further studies showed that the PlaS belonged to the ankyrin family (ANK repeat), consistent with the previous experimental results. The analysis in the NCBI database showed that the phospholipase A1 auxiliary protein included four typical ANK repeats at amino acids sequences 46-76, 78-109, 111-142, 145-176 of PlaS, as shown in Figure 2A.

Fig. 2: Bioinformatics analysis results of PlaA1 and PlaS. (A) Auxiliary protein amino acids sequence diagram. (B) Tertiary structure diagram of auxiliary protein. (C) Three-dimensional structure prediction diagram and active center site of PlaA1. (D) The DoG-SiteScorer calculated the simulated PlaA1 "catalytic pocket". (E) Molecular docking diagram of PlaS and phospholipase A1.

Three-dimensional structure modeling

According to Discovery Studio 3.5 software, the 3D structure of PlaS was simulated and shown in Figure 2B. It showed that the overall structure of PlaS presented a slightly curved envelope shape. Each ANK repeat consists of two α-helices and two β-folds, forming a highly hydrophobic region internally. Furthermore, the prediction results of *phyre2*showed that the matching ratio of PlaA1 to the fold d3tgla reached 98%, and the matching ratio of PlaS to the fold c4cj9A reached 100%. Thus modeling and molecular docking can be performed. In addition, the sequence "GSHXGG" is a recognition sequence of that triacylglycerol lipase family and is the core catalytic site in the "Ser-His-Asp" catalytic triad. Through analysis of the spatial structure of PlaA1, we found that this conservative sequence was wrapped inside the protein space, and its 3D structure was shown in Figure 2C.

DoGSiteScorer is an online analysis software for calculating the "catalytic pocket". It showed a "catalytic pocket" formed by Leu197-Ser249 outside the space of PlaA1 after calculating, as shown in Figure 2D. The information of the "catalytic pocket" is shown in Table 1.

Table 1. "Catalytic pocket" information sheet for PlaA1.

Name	Item	Value
Size and Shape descriptors	Volume[Å^3]	1263.17
	Surface[Å^2]	1790.04
	Depth[Å]	23.87
Functional group descriptors	Hydrogen bond donors	17
Amino acid composition	Hydrogen bond acceptors	74
	Hydrophobic interactions	98
	Hydrophobicity ratio	0.52
	Apolar amino acid ratio	0.49
	Polar amino acid ratio	0.41
	Positive amino acid ratio	0.04
	Negative amino acid ratio	0.06

Molecular docking of PlaA1 and PlaS

The molecular docking of protein-protein was performed by the ZDOCK program. By optimizing and scoring, the optimal predicted binding configuration, shown in Figure 2E, was selected as the docking configuration of PlaA1 and PlaS. In the process of interaction, the interface amino acid of protein was crucial for predicting the protein binding site. By simulating the molecular docking of PlaA1 and PlaS, there were 29 amino acids of PlaA1 and 30 amino acids of PlaS to participate in the binding shown in table 2. The binding sites with larger contact areas between PlaA1 and PlaS were PlaA1$^{\mathrm{Phe186}}$ and PlaA1$^{\mathrm{Lys238}}$, reaching 81.5464Å^2 and 73.2219Å^2, respectively.

Table 2. Amino acid types and contact area at the interface between PlaA1 and PlaS.

Amino acid binding site on PlaA1	Area(Å^2)	Amino acid binding site on PlaS	Area(Å^2)
Phe186	81.5464	Asn228	62.4389
Lys238	73.2219	His250	61.0948
Ala235	45.2023	His117	52.5794
Lys183	43.8116	Asp239	52.5092
Asp188	42.9515	Lys188	49.4284
Asp232	41.7991	Tyr218	42.6094
Ala184	41.6193	Ala151	35.9948

Amino acid binding site on PlaA1	Area(Å²)	Amino acid binding site on PlaS	Area(Å²)
Ile121	38.7777	Pro223	33.6571
Val185	29.4011	Leu155	32.6841
Asp141	27.6104	Val154	27.8932
Gly187	24.2322	Thr222	23.6619
Ile231	17.2322	Ala224	22.9488
Thr209	16.9793	Gln227	22.554
Pro233	11.3448	Asn113	22.5273
Phe119	8.00322	Ser139	20.58
Leu150	7.93828	Ala147	18.4521
Ala242	7.75555	His184	17.4887
Glu241	6.08212	Thr148	12.7114
Asp122	5.57528	Leu231	12.6936
Lys237	4.56579	Ile121	12.4643
Tyr144	3.60079	Ala187	12.17
Leu190	3.60079	Ala120	11.0265
Leu229	3.38873	Gln84	10.1369
Asn140	2.78764	Leu116	9.69443
Ala234	2.78764	Val80	8.03253
Leu207	2.53422	Thr249	7.60265
Ala182	1.26711	Arg158	5.30144
Arg228	0.760269	Ala156	5.03637
Lys180	0.506843	Glu230	2.39861
-	-	His225	2.1444

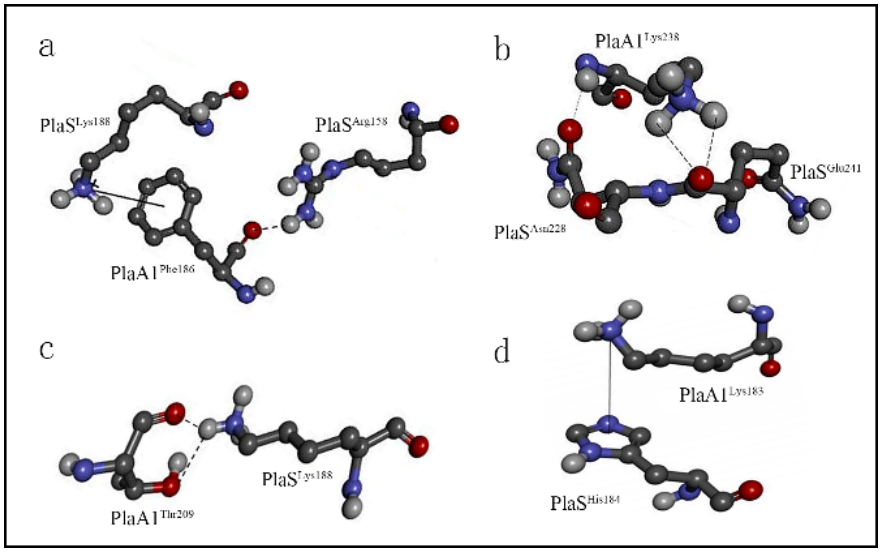


Table 3 lists non-covalent bonds interactions between PlaA1 and PlaS. In PlaA1, Phe186 formed both Pi and hydrogen bonds, as shown in Figure 1F(a). The contact area of Lys238 at the binding interface was also relatively large. It participates in forming three hydrogen bonds, as shown in Figure 1F(b). In PlaS, the amino acids forming hydrogen bonds were Asn228, Gln227, Ala151, Arg158, and Lys188. Non-covalent bond of PlaS^{Lys188} and PlaS^{His184} are shown in Figure 3.

Fig. 3: Schematic of non-covalent bonds residual interactions between PlaA1 and PlaS.

Table 3. Non-covalent bond interactions of PlaA1 and PlaS.

PlaA1	PlaS	Interaction Constituents	Distance (Å)	Type
Lys183	His184	Lys183 - His184	4.6253	Interface Pi-Cation
Phe186	Lys188	Phe186 - Lys188	3.7556	
Lys238	Asn228	Lys238 - Asn228	1.8768	Hydrogen Bonds
	Glu241	Lys238 - Gln227	2.1198	
	Glu241	Lys238 - Gln227	2.0829	
Ala184	Ala151	Ala151 - Ala184	2.4559	
Phe186	Arg158	Arg158 - Phe186	1.9218	
Thr209	Lys188	Lys188 - Thr209	2.3913	
Thr209	Lys188	Lys188 - Thr209	1.7214	

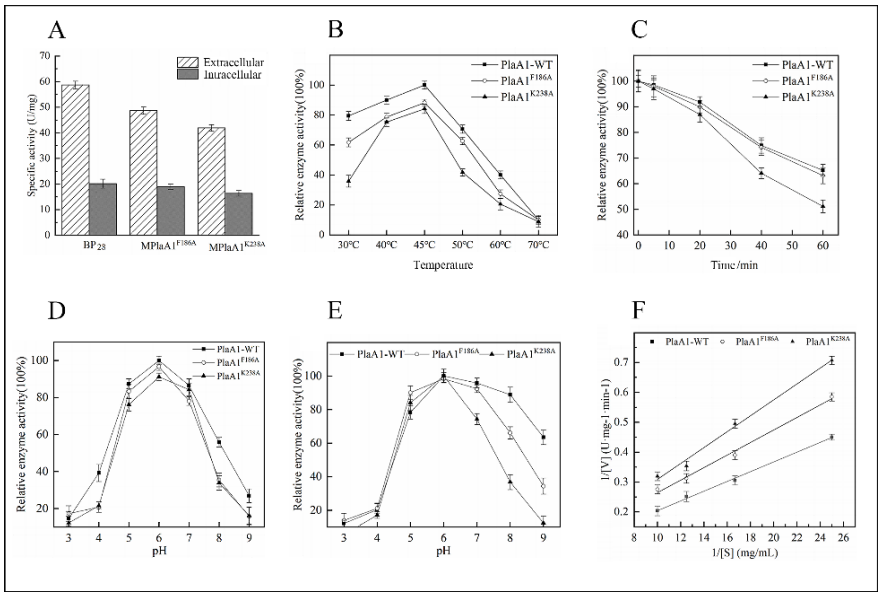
Enzymological properties of PlaA1^{F186A} and PlaA1^{K238A}

MPlaA1^{F186A} and MPlaA1^{K238A} are the names of the strains which mutated Phe186 and Lys238 into Ala, respectively. BP_{28} is used as the original strain. The extracellular specific enzyme activity of phospholipase A1 produced by mutant strain MPlaA1^{F186A} and MPlaA1^{K238A} was 48.67U/mg and 41.9U/mg, respectively, as shown in Figure 4A. Compared with the extracellular specific enzyme activity of phospholipase A1 produced by non-truncated strain BP_{28} (58.68 U/mg), the extracellular specific enzyme activity of phospholipase A1 produced by mutant strain MPlaA1^{F186A} and MPlaA1^{K238A} was 17% and 28.6%, respectively. At the same time, there was insignificant difference in the intracellular enzyme activities of each strain. The change of Lys 238 of PlaA1 had a more significant effect on the enzyme activity from the enzyme activity data.

Optimum Temperature and Thermal Stability of PlaA1^{F186A} and PlaA1^{K238A}

As shown in Figure 4B, compared with the optimum temperature of the enzyme PlaA1-WT produced by the original strain BP_{28}, mutant PlaA1^{F186A} and PlaA1^{K238A}remained unchanged, both of which were 45. When the reaction temperature was below 45, the enzyme activity produced by the starting strain BP_{28} decreased slowly, while the mutant PlaA1^{K238A} decreased rapidly especially the temperature was below 40. When the temperature was above 45, the enzyme activity of mutant PlaA1^{K238A} began to decline sharply. As shown in Figure 4C, the mutant enzyme PlaA1^{K238A} exhibited the worst thermal stability. When the incubation time was more than 20min, the enzyme activity was decreased significantly. However, the thermal stability differences between the enzyme PlaA1-WT produced by the original strain BP_{28} and the mutant enzyme PlaA1^{F186A}were insignificant.

Optimum pH and pH Stability of PlaA1^{F186A} and PlaA1^{K238A}



In order to explore the optimal pH of PlaA1^{F186A} and PlaA1^{K238A}, the enzyme activity of phospholipase A1 produced by BP_{28} at the optimal reaction pH was defined as 100%, and the relative enzyme activities of samples with other pH values were calculated. As shown in Figure 4D, the optimal pH values of the mutant PlaA1 ^{F186A} and PlaA1^{K238A} were unchanged from that of PlaA1-WT, both of which were pH6. The relative enzyme activities of the mutant PlaA1^{F186A} and PlaA1^{K238A} were relatively stable when pH was between 5 and 6, as shown in Figure 4E. When pH5, the relative enzyme activity of PlaA1^{K238A}was 75.29%, while when pH8, the activity decreased sharply to 36.78%, indicating that PlaA1^{K238A} was acidic relative to PlaA1-WT. The pH stability of PlaA1^{F186A} was between that of PlaA-WT and PlaA1^{K238A}. When pH was more significant than 7, the relative enzyme activity of mutant PlaA1^{F186A} also decreased significantly. In general, the pH stability of mutant PlaA1^{K238A} was poor.

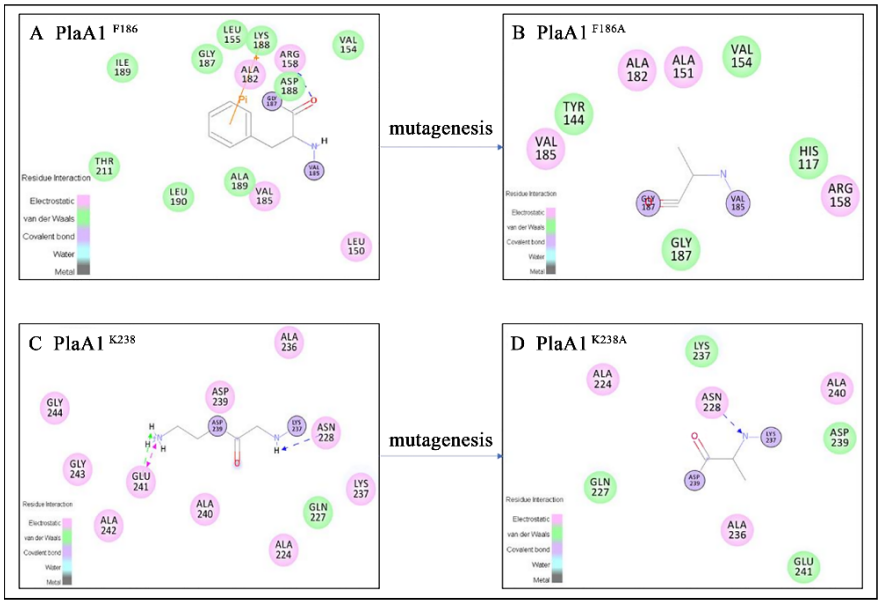


Fig. 4: Research results of enzymatic properties. **(A)** Specific enzyme activity of each bacterium in and outside the cell respectively.**(B)** Optimum temperatures for PlaA1-WT, PlaA1^{F186A} and PlaA1^{K238A}.**(C)** Thermal stability of PlaA1-WT, PlaA1^{F186A}and PlaA1^{K238A}. **(D)** Optimum pH of PlaA1-WT, PlaA1^{F186A} and PlaA1^{K238A}.**(E)** pH stability of PlaA1-WT, PlaA1^{F186A} and PlaA1^{K238A}. **(F)** Km determination of each mutant strain phospholipase A1 by Lineweaver-Burk plot.

Fig. 5: Schematic diagram of hydrogen bond interaction with PlaS before and after mutation of PlaA1. **(A)** is PlaA1^{F186}. **(B)** is PlaA1^{F186A}. **(C)** is PlaA1^{K238}. **(D)** is PlaA1^{K238A}.

Enzymatic kinetics

Figure 4F shows that Michaelis constant of phospholipase A1 of each mutant strain. Table 4 and 5 show the enzymatic kinetic parameters. The Km value of PlaA1^{F186A} was insignificantly different from that of PlaA1-WT, while PlaA1^{K238A} was 1.6 times higher than PlaA1-WT. While the catalytic efficiency (kcat/Km) of mutant enzymes reduced by 29.53% and 47.5% compared with that of PlaA1-WT, respectively. This experiment showed that after the phenylalanine at position 186 and lysine at position 238 were mutated into nonpolar alanine, the affinity of PlaA1 to the substrate was weakened, and the catalytic efficiency and enzyme activity were also weak. Among them, the mutant enzyme PlaA1^{K238A} showed significant differences.

Table 4. Enzyme kinetic equation table of each strain.

Name	**Dynamical equation**	**R^2**
PlaA1-WT	y=0.0162x+0.0426	0.99854
PlaA1 ^{F186A}	y=0.02099x+0.05422	0.99664
PlaA1 ^{K238A}	y=0.02662x+0.04236	0.99648

Table 5. Kinetic parameters of each strain.

Kinetic parameter	Km (mg/mL)	Vmax (U·mg-1·min-1)	Kcat/Km (mL/(mg·s))
PlaA1-WT	0.3798±0.0022	23.45±1.21	1083.21±87.34
PlaA1 ^{F186A}	0.3871±0.0138	18.44±0.66*	763.40±56.55*
PlaA1 ^{K238A}	0.6284±0.0283**	23.61±1.06	569.22±53.64**

Prediction of spatial structure

The spatial structure of mutant enzymes PlaA1^{F186A} and PlaA1^{K238A} was predicted, and the "catalytic pockets" were calculated. From the perspective of spatial structure, mutant enzymes did not undergo significant changes with the wild-type enzyme. Based on the calculated "catalytic pocket" analysis data, as shown in table 6. The volumes and surface areas of mutant enzymes became significantly smaller, with the mutant enzyme PlaA1^{K238A} changing significantly and the "catalytic pocket" deepening. The Lys238 of phospholipase A1 is just one of the constituent amino acid residues in the "catalytic pocket" of PlaA1. When PlaA1 interacts with PlaS, Lys238 plays an indispensable role in affecting the size of the "catalytic pocket" of PlaA1.

After analysis by molecular docking software, we mutated the phenylalanine at position 186 of PlaA1 into alanine, and the π-bond and hydrogen bonds provided by Phe186 disappeared, as shown in Figure 3A and B. After lysine at position 238 of PlaA1 was mutated into alanine, the number of hydrogen bonds was reduced from three to one, as shown in Figure 3C and D.

Table 6. Size and shape of "catalytic pocket" for PlaA1 and mutant enzymes.

Size and Shape descriptors	PlaA1	PlaA1^{F186A}	PlaA1^{K238A}
Volume[Å^3]	1263.17	1154.05	1156.16
Surface[Å^2]	1790.04	1621.1	1586.96
Depth[Å]	23.94	23.87	24.74

Discussion

With the significant role of microbial fermentation in industrial production, more and more researchers have turned their attention to the production of phospholipase A1 by microbial fermentation. At present, the phospholipase A1 sold on the market is mainly produced by*Aspergillus niger* , but due to the complex purification process, it has become crucial to clone and express phospholipase A1 in prokaryotes. Since 1992, researchers have expressed the phospholipase A1 gene by modifying the dual-start subsystem of *E. coli* , and heterologous expression of phospholipase A1 has not met the needs of industrial production [4, 20]. Later, some scholars successively increased the enzyme activity by adding trace elements, optimizing the culture scheme, and mutation breeding [5, 19, 21]. With the continuous development of technology, immobilized enzyme technology has been applied to phospholipase A1 in recent years, and the activity and stability of phospholipase A1 have been further improved at this level [22]. Studies have found that the extracellular expression of phospholipase A1 is closely related to the auxiliary protein encoded by its downstream gene. The auxiliary protein of phospholipase A1 promotes the extracellular expression of phospholipase A1, but it does not have phospholipase activity.

In order to further understand the physiological function of phospholipase A1, we analyzed the structure of phospholipase A1 auxiliary protein from *Serratia marcescens* and found that the auxiliary protein belongs to the ankyrin repeat family [23]. Ankyrin repeat (ANK), as the main element constituting the structure

of anchorin, is a universal protein sequence motif in organisms [24]. Biochemical structural characteristics indicate that these repetitive sequences are accumulated side by side as basic modules to form a modular and specific infrastructure with proteins binding interface. Compared with other protein interaction domains (such as SH2 or SH3), these modular repetitions generally do not recognize any specific amino acid sequence or structure[25]. Instead, they form elongated surfaces of varying sizes depending on the number of repetitions. The specificity of protein ligands is determined by changes in the active residues of these elongated surfaces of varying sizes. Among the characterized ankyrin repeat, a unified feature is that they usually mediate specific protein interactions [26-31]. The study of auxiliary proteins will provide a new idea for the modification of phospholipase A1. However, molecular docking is a well-known bioinformatics theoretical simulation method, and it has attracted extensive attention to study the interaction between molecules (such as ligands and receptors), predict their binding mode and affinity by computer platform, accurately predict the ligand-drug structure at the receptor-binding site, and accurately estimate the binding strength [32-36]. This study aimed to provide a new theory for explaining how the auxiliary protein promotes the extracellular secretion mechanism of phospholipase A1 and some directions for future research in the selection or transformation of high-yielding strains of phospholipase A1 by predicting the tertiary structure and simulating molecular docking.

The data obtained in structural simulation and molecular docking showed that, since phospholipase A1 also belongs to the triacylglycerol lipase family, its active central site is covered by a "lid" and a "β9" ring in the lipase family. We have learned that serine residues of active sites are wrapped inside the molecule, while the outside is a "Lid" formed by α-helix. When the "Lid" is opened, an electrophilic region will be formed around Ser to expose the hydrophobic residue, thus enhancing the affinity with the substrate to interact with the core catalytic amino acids. At the same time, the existence of the "Lid" maintains the stability of the intermediate product in the catalytic process[37-40]. The 3D structure showed that the overall structure of PlaS presented a slightly curved envelope shape. Each ANK repeat of PlaS consists of two α-helices and two β-folds, forming a highly hydrophobic region internally [41-44]. Due to the enrichment of the α-helix, the internal junctions of the auxiliary protein PlaS form highly hydrophobic regions, which facilitate binding to the target protein.

By simulating the molecular docking of PlaA1 and PlaS, on the binding interface of the two, there were 29 amino acids in PlaA1 and 30 amino acids in PlaS. Binding sites with larger contact areas between PlaA1 and PlaS were PlaA1^{Phe186} and PlaA1^{Lys238}. PlaA1^{Phe186}participated in both the formation of π-bonds and hydrogen bonds. PlaA1^{Lys238} participated in forming three hydrogen bonds. Chemical bonds are essential to maintain the interaction between receptor and ligand. A study showed that the increase of hydrogen bonds contributed to the stability of the enzyme [45]. So, in the interaction between PlaA1 and PlaS, hydrogen bonds and π-bonds played significant roles. In addition, the "catalytic pocket" of phospholipase A1 contains the "lid", and the combination of PlaA1 and PlaS may have caused the change of the "catalytic pocket" at the active site.

By analyzing the enzymatic properties of the mutant enzymes PlaA1^{F186A}, PlaA1^{K238A}, and the primitive enzyme, we found that the optimal temperature and pH of the mutant enzymes PlaA1^{F186A} and PlaA1^{K238A} did not change significantly, but the thermal and pH stability of PlaA1^{K238A} were poor. The kinetic parameters showed that after the Phe186 and Lys238 were mutated into nonpolar alanine, the affinity of mutant enzymes to substrates was weakened, both the catalytic efficiency and enzyme activity also decreased. Among them, the mutant PlaA1^{K238A} showed significant differences. According to the preliminary docking analysis, seven hydrogen bonds interact between PlaA1 and PlaS in the binding process, while PlaA1^{Lys238} formed three hydrogen bonds. Therefore, Lys238 provided hydrogen bonds interact with a relative specific gravity in the binding process with PlaS. The existence of hydrogen bonds plays a vital role in maintaining the stability of proteins. PlaA1^{Lys238} is also one of the constituent amino acid residues in the "catalytic pocket" of PlaA1. When PlaA1 interacts with PlaS, Lys238 plays a key role in affecting the size of the "catalytic pocket" of PlaA1.

The bioinformatics software was used to calculate the hydrophobic distraction, "catalytic pocket", and the

structural analysis of the mutant amino acid sites of PlaA1^{F186A} and PlaA1^{K238A}. After the Phe186 of PlaA1 is mutated into Ala, the "catalytic pocket" becomes smaller, and the π-bond and the hydrogen-bond disappeared. It has been reported that the total number of hydrogen bonds plays an important role in the stability of the protein structure [46-47]. After the Lys238 of PlaA1 was mutated into Ala, the "catalytic pocket" was significantly smaller, the hydrophobicity was enhanced, and the number of hydrogen bonds formed was also reduced from three to one, which was consistent with the experimental results. The site-directed mutagenesis results confirmed that PlaA1^{Lys238} was the key binding site for PlaA1 and PlaS. It is worth mentioning that some people have found a new auxiliary site in phospholipase A1 of Thai Banded Tiger Wasp (Vespa affinis), which is beneficial to enhance the enzymatic reaction[48]. The results of this study were also instructive to our research.

In conclusion, we can infer that the π-bonds and hydrogen bonds generated by the interaction of phospholipase A1 with PlaS are the reasons for some changes in the "catalytic pocket" of phospholipase A1, and these non-covalent bonds are the critical factors for the regular fold expression of phospholipase A1 and the performance of high enzyme activity and stability. However, all aspects of this potential mechanism need to be further studied to facilitate the further rational design and modification of phospholipase A1.

Conclusion

In summary, in this work, we used the molecular docking software DS to dock PlaA1 and PlaS molecularly and speculated that the binding of PlaA1 to PlaS may have contributed to the change of the "catalytic pocket" at the active site. The analysis of amino acids at the binding interface indicated that the amino acids with relatively large contact areas were PlaA1^{Phe186}, PlaA1^{Lys238}, PlaS^{Asn228}, and PlaS^{His250}. In the interaction with PlaS, PlaA1^{Phe186} was involved in both π-bonds and hydrogen-bonds formation, while PlaA1^{Lys238} was involved in formatting three hydrogen bonds. On this basis, we screened Phe186 and Lys238 of phospholipase A1 and mutated them to nonpolar Ala. The results of the targeted mutation verified PlaA1^{Lys238} as the key binding site for PlaA1 and PlaS. The enzymatic properties of the mutated enzyme were investigated, and bioinformatics analysis revealed that Phe186 and Lys238 of PlaA1 provide non-covalent bonds that facilitate the natural folding of phospholipase A1 during the interaction of phospholipase A1 with its auxiliary protein, improving enzymatic activity and maintaining stability. Therefore, the follow-up work of this study will build on this research work to perform saturation or combination mutagenesis on the binding region of PlaA1 and PlaS to obtain lipases with higher catalytic activity. This work will also facilitate the discovery of the mechanism of action of PlaS on the high enzymatic activity and stability performance of phospholipase A1, bringing new explanations for the function of the ankyrin repeat family.

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